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Novel microorganisms capable of assimilating lactose.

Described are coryneform microorganisms capable of assimilating lactose which carry a recombinant DNA capable of conferring the ability to assimilate lactose on coryneform microorganisms, and a process for producing L-amino acids which comprises culturing said coryneform microorganism capable of assimilating lactose in a culture medium containing lactose to form an amino acid, and recovering said amino acid accumulated in the culture broth. Further, the invention relates to a method for preparing recombinant plasmids containing a DNA fragment essential to the expression of a gene in coryneform microorganisms.

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Novel Microorganisms Capable of Assimilating Lactose

Background of the Invention

As the processes for producing amino acids by using glutamic acid-producing coryneform bacteria
5 belonging to the genus Corynebacterium or Brevibacterium, there have been known processes for
producing glutamic acid by using wild-type strains of such genus and processes for producing various
amino acids by using mutant strains derived from wild-type strains of such genus ["Amino Acid Fermenta-
tion, (I) and (II)" Association of Amino Acid and Nucleic Acid (Kyoritsu Shuppan, 1972); and "Amino Acid
Fermentation" by Aida, Takinami and Chibata (Gakkai Publishing Center, 1986)]. Further, plasmid vectors for
10 glutamic acid-producing coryneform bacteria [Japanese Published Unexamined Patent Application No.
134500/82 (European Patent No. 58889, U.S. Patent No. 4617267), Japanese Published Unexamined Patent
Application No. 183799/82 (European Publication No. 63763, U.S. Patent No. 4500640) and Japanese
Published Unexamined Patent Application No. 105999/83 (European Publication No. 82485, U.S. Patent
Application Serial No. 668674)], and methods for the transformation of these bacteria [Japanese Published
15 Unexamined Patent Application No. 186492/82 (European Publication No. 63764, U.S. Patent No. 4683205)
and Japanese Published Unexamined Patent Application No. 186489/82 (European Publication No. 64680,
U.S. Patent No. 4681847)] have been recently developed. As a result, recombinant DNA technology has now
become applicable to strains of the genus Corynebacterium or Brevibacterium, whereby new aspects on the
effective utilization of these microorganisms have been opened.
20 The recombinant DNA technology has made it possible to clone a gene responsible for an enzyme that
catalyzes the rate-limiting reaction in the biosynthetic pathway of an amino acid-producing microorganism
and to introduce a recombinant DNA containing the cloned gene into a host microorganism, thereby
enhancing the activity of said rate-limiting enzyme. There have been already known the cases in which
amino acid productivity of coryneform bacteria has been increased by introduction of a recombinant DNA
25 containing a cloned gene derived from a coryneform bacterium or from a microorganism of different
species such as Escherichia coli [Japanese Published Unexamined Patent Application No. 126789/83
(European Publication No. 88166, U.S. Patendt Application Serial No. 787010), Japanese Published Unex-
amined Patent Application Nos. 156292/84, 156294/84, 24192/85, 34197/85, 30693/85 and 66989/85 (U.S.
Patent Application Serial Nos. 073888, 580814, 613209, 631648, 631649 and 646512) (European Publication
30 No. 136359)]. Fermentation of these microorganisms of the genus Corynebacterium or Brevibacterium for the
production of various amino acids is carried out by using, as the main carbon source, glucose, fructose,
sucrose, maltose, acetic acid, ethanol, lactic acid or substances containing the same (for example, starch
hydrolyzate and cane molasses), which these strains are hereditarily able to assimilate.
.Attempts have also been made to use lactose as a carbon source for the production of amino acids by
35 microorganisms belonging to the genus Corynebacterium or Brevibacterium. However, lactose cannot be
employed as the main carbon source because the glutamic acid-producing coryneform bacteria have no
ability to assimilate lactose [Nogeikagaku Kaishi, 39, 328 (1965)]. The mixed culture is a known example of
the process for producing amino acids by fermentation in which lactose is used as a carbon source. In the
process, a lactose-assimilating bacterium (e.g., a lactobacillus) and an amino acid-producing bacterium
40 belonging to the genus Corynebacterium or Brevibacterium are simultaneously cultured in a medium
containing lactose, whereby lactose is assimilated into lactic acid by the former, and the latter assimilates
lactic acid to produce glutamic acid, lysine or valine (Japanese Published Unexamined Patent Application
Nos. 174095/82, 170194/82 and 208993/82).
Whey which is generated in large quantities as liquid waste from the cheese and casein manufacturing
45 processes generally contains about 5% lactose and about 1% proteins. Although a part of it is used as food,
animal feed additives and fertilizers, it is discarded for the most part. This is undesirable not only from the
viewpoint of utilization of resources but also from that of environmental protection from pollution [The Food
Industry, 18 No. 12, 20 (1975)]. Hence, use of whey as a fermentation material, if possible, would be of great
industrial significance, as it would contribute to the effective utilization of resources and the environmental
50 protection. The aforementioned mixed culture for the production of glutamic acid and other amino acids is
intended for the utilization of lactose contained in whey, but it suffers various disadvantages. If lactic acid
fermentation by the lactose-assimilating bacterium progresses rapidly, the culture broth becomes acidic,
whereby the amino acid fermentation by the coryneform bacterium is inhibited. Accordingly, the amounts of
the two strains to be inoculated and culture conditions must be elaborately determined according to the
lactic acid-assimilating activity of the amino acid-producing strain belonging to the genus Corynebacterium

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or Brevibacterium which is used in the mixed culture. Thus, intricate operations and strict culture control are required. Under the circumstances, the present inventers tried to confer, by recombinant DNA technology, the ability to assimilate lactose on strains of Corynebacterium or Brevibacterium which genetically lack this ability in order to develop a new fermentation process for producing various amino acids in which a microorganism of Corynebacterium or Brevibacterium alone is cultured in a medium containing a lactose-containing substance such as whey as a carbon source.

This improvement of amino acid-producing bacteria by introduction of a recombinant DNA is based on the amplification effect of enzyme genes which are capable of expression in a coryneform bacterium. The expression of these genes takes place under the control of expression regulatory region inherent to the donor microorganism of these genes. Therefore, in some cases, the expression of the genes in a host microorganism may not be so efficient and the amino acid productivity can not be markedly improved. The expression efficiency of introduced genes must be enhanced in a suitable way to ensure a high amino acid productivity.

It is generally accepted that a promoter region necessary for the initiation of transcription and a ribosome-binding sequence essential to messenger RNA translation must be positioned upstream of a structural gene in order to ensure the expression of the gene in a microorganism, and that expression efficiency of the gene depends mainly on the activity of the promoter. Since the promoter region and the ribosome-binding sequence are considered to have species-specific base sequences, efficient expression of a desired structural gene would be achieved by disposing the structural gene downstream of a highly active promoter region and a proper ribosome-binding sequence derived from the host microorganism. Such an efficient gene expression system has already been established for Escherichia coli whose mechanism of gene expression has been reported in detail [Nakamura, K., Kagaku To Seibutsu, 20, 47 (1982)] and for Bacillus subtilis on which detailed genetic analysis has also been made (Horinouchi, S., Proteins/Nucleic Acids/Enzymes, 28, 1468 (1983); Goldfarb, D.S. et al., Nature, 293, 309 (1981)].

As a method for preparing a promoter region derived from a coryneform bacterium, there has been reported the use of a promoter-probing vector carrying a promoter-less chloramphenicol resistance gene which is used for detection of promoter activity (Japanese Published Unexamined Patent Application No. 124387/86). This vector contains the chloramphenicol resistance gene of Escherichia coli except its promoter region, that is, the structural gene and ribosome-binding sequence of that gene, and further has a promoter cloning site upstream therefrom. A DNA fragment containing the promoter region of a coryneform bacterium can be obtained by inserting a DNA fragment derived from the coryneform bacterium into this cloning site and selecting a recombinant plasmid which can confer chloramphenicol resistance on coryneform bacteria. It is considered that transcription of the chloramphenicol resistance gene in the recombinant plasmid thus obtained starts at the promoter region derived from the coryneform bacterium to form a messenger RNA, and translation of the messenger RNA is started by the function of the ribosome-binding sequence originated from the sequence of Escherichia coli chloramphenicol resistance gene, leading to the expression of the gene. However, the species-specificity in the gene-expression control region mentioned above suggests that insertion of a highly active promoter derived from a coryneform bacterium does not necessarily ensure efficient gene expression, because the ribosome-binding sequence of Escherichia coli might be unsuitable for translation in coryneform bacteria and hence translation might be rate-limiting in the expression process. Thus, it would be desirable to clone a DNA fragment having both a transcription initiation site and a translation initiation site in order to obtain a gene-expression control region which ensures efficient gene expression in coryneform bacteria. However, no effective method has so far been known to isolate such a DNA fragment.

It has been found that microorganisms belonging to the genus Corynebacterium or Brevibacterium can be imparted with the ability to assimilate lactose by introducing the genetic information derived from E-scherichia coli lactose operon into a strain of the genus Corynebacterium or Brevibacterium by recombinant DNA technology and expressing the introduced information.

Further, intensive studies have been made to isolate the promoter region and the ribosome-binding sequence responsible for the initiation of transcription and translation, respectively, which are both essential to the expression of a gene in coryneform bacteria, from a chromosomal DNA. As a result, a plasmid vector has been constructed which has autonomous replicability in a coryneform bacterium, and contains a DNA fragment carrying structural genes coding for β-D-galactosidase, or coding for β-D-galactosidase and β-galactoside permease derived from Escherichia coli lactose operon, and can be used to clone a DNA fragment responsible for the gene expression control in coryneform bacteria. The present invention has been accomplished based on these findings.

Summary of the Invention

The present invention relates to coryneform microorganisms capable of assimilating lactose which carry a recombinant DNA containing genetic information derived from Escherichia coli lactose operon and
5   capable of conferring the ability to assimilate lactose on microorganisms belonging to the genus Corynebacterium or Brevibacterium by the expression of the information; and to a fermentation process for producing L-amino acids which comprises culturing said coryneform microorganism capable of assimilating lactose in a culture medium containing lactose to form an amino acid, and recovering said amino acid accumulated in the culture broth. Hence, the present invention is related to the bioindustry, particularly to
10   the manufacture of amino acids useful in the pharmaceutical, food and animal feed industries.

The present invention further relates to a method for preparing recombinant plasmids containing a DNA fragment essential to the expression of a gene in coryneform bacteria belonging to the genus Corynebacterium or Brevibacterium. Such recombinant plasmids are useful for obtaining DNA fragments which have functions necessary to enable or enhance the expression of desired genes in coryneform bacteria, and
15   thereby for enabling the improvement of metabolite productivity of coryneform bacteria, the conferment of new functions on coryneform bacteria and the production of useful enzymes and physiologically active proteins in coryneform bacteria.

20   Brief Description of the Drawings

Figure I illustrates the steps for constructing pCGIII.
Figure 2 illustrates the steps for constructing pE'lacI, in which the solid box represents the DNA fragment derived from Escherichia coli lactose operon.
25   In both of these figures, the sizes of plasmid DNAs are expressed in kilobase (kb).

Detailed Description of the Invention

30   The present invention relates to a coryneform microorganism capable of assimilating lactose and carrying a recombinant DNA which confers the ability to assimilate lactose on a microorganism belonging to the genus Corynebacterium or Brevibacterium, said recombinant DNA comprising (a) a part or all of the coding region of the structural genes for β-D-galactosidase and β-galactoside permease derived from Escherichia coli lactose operon, and (b) a DNA fragment derived from a microorganism belonging to the
35   genus Corynebacterium or Brevibacterium and enabling expression of foreign genes in strains belonging to the genus Corynebacterium or Brevibacterium, which is positioned upstream of the coding region of said structural genes; and to a process for producing amino acids which comprises culturing said coryneform bacterium capable of assimilating lactose in a culture medium containing lactose to produce an amino acid, and recovering said amino acid accumulated in the culture broth.
40   The utility of the invention is to allow the production of various L-amino acids by fermentation using, as a carbon source, lactose contained in whey which is produced in large quantities as a waste in the cheese and casein manufacturing processes and now discarded for the most part. This process for conferring the ability to assimilate lactose on coryneform bacteria belonging to the genus Corynebacterium or Brevibacterium, which are inherently unable to assimilate lactose, by recombinant DNA technology, and thereby
45   enabling the production of various amino acids by direct fermentation using, as a carbon source, lactose or lactose-containing substance such as whey has been first established by the present invention.

Amino acids that can be produced by the process established by the present invention include glutamic acid, glutamine, lysine, threonine, isoleucine, valine, leucine, tryptophan, phenylalanine, tyrosine, histidine, arginine, ornithine, citrulline and proline.
50   The present invention also relates to a method for preparing a recombinant plasmid containing a DNA fragment responsible for the initiation of transcription and translation necessary for the expression of a gene in a microorganism belonging to the genus Corynebacterium or Brevibacterium, which comprises (a) inserting a DNA fragment derived from a microorganism belonging to the genus Corynebacterium or Brevibacterium into a plasmid at a restriction enzyme cleavage site, said plasmid having autonomous
55   replicability in a microorganism of the genus Corynebacterium or Brevibacterium and containing selectable genetic markers, a DNA fragment derived from Escherichia coli lactose operon and carrying the coding region of structural genes necessary for the expression of β-D-galactosidase activity, and restriction enzyme cleavage sites positioned just before said coding region; (b) transforming a strain of the genus Corynebac-

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terium or Brevibacterium by using the recombinant plasmid thus prepared to obtain a transformant having the ability to assimilate lactose; and (c) isolating the recombinant plasmid from said transformant.

The plasmid used for cloning the DNA fragment responsible for the initiation of transcription and translation in a coryneform bacterium may be prepared by any method, so long as it has autonomous
5 replicability in a strain of the genus Corynebacterium or Brevibacterium, and contains selectable genetic markers, the structural genes coding for β-D-galactosidase, or coding for β-D-galactosidase and β-galactoside permease derived from Escherichia coli lactose operon, and restriction enzyme cleavage sites positioned just before said structural genes. For example, it may be prepared by isolating a DNA fragment containing lacZ (structural gene for β-D-galactosidase) and lacY (structural gene for β-galactoside per-
10 mease) from lactose operon of Escherichia coli KI2 strain, and ligating it to a plasmid vector autonomously replicable in a strain of the genus Corynebacterium or Brevibacterium and having a suitable selection marker.

Any strain belonging to the genus Corynebacterium or Brevibacterium and having an ability to produce an amino acid may be used as the host microorganism in the present invention. Preferred examples are the
15 strains listed below and mutants derived therefrom.

Corynebacterium glutamicum   ATCC I3032
Corynebacterium acetoacidophilum   ATCC I3870
Corynebacterium herculis   ATCC I3868·
20 Corynebacterium lilium   ATCC I5990
Brevibacterium divaricatum   ATCC I4020
Brevibacterium flavum   ATCC I4067
Brevibacterium immariophilium   ATCC I4068
Brevibacterium lactofermentum   ATCC I3869
25 Brevibacterium thiogenitalis   ATCC I9240

Wild-type strains may be used for producing glutamic acid and glutamine, but it is preferred to use mutants with improved productivity (for example, the mutants described in Japanese Published Unexamined Patent Application Nos. 66990/85 and I48094/80). Various amino acid-producing strains constructed by
30 known mutagenesis or recombinant DNA technology may be used for producing other amino acids.

The genetic information on lactose assimilation can be obtained from the lactose operon relating to this function in Escherichia coli KI2 strain. The lactose operon has been investigated in most detail among the genes of Escherichia coli, and its structure and expression mechanism have been elucidated almost completely ["The Lactose Operon" by J.R. Beckwith; Cold Spring Harbor Laboratory (1982)]. The lactose
35 operon contains three structural genes, i.e. lacZ, lacY and lacA which are located in that order and are transcribed as a single transcription unit. The genes lacZ, lacY and lacA code for β-D-galactosidase, β-galactoside permease and galactoside acetyltransferase, respectively, and β-D-galactosidase and β-galactoside permease are essential to assimilation of lactose. Lactose is taken in through a cell membrane by the action of β-galactoside permease, and hydrolyzed into galactose and glucose by the action of β-D-
40 galactosidase, and the galactose and glucose thus formed are then metabolized through the general metabolic pathway.

Conferment of the ability to assimilate lactose on a strain of the genus Corynebacterium or Brevibacterium depends on whether or not the genes coding for β-D-galactosidase and β-galactoside permease derived from Escherichia coli lactose operon can be expressed in that strain. Actually, however, when a
45 recombinant plasmid which contains lactose operon including the transcription and translation signals derived from Escherichia coli is used, its genetic information is expressed in a strain of the genus Corynebacterium or Brevibacterium but feebly, failing to confer the ability to assimilate lactose on the strain. This fact indicates that the expression of a gene must be sufficiently intense in order to confer the ability to assimilate lactose on a strain of the genus Corynebacterium or Brevibacterium. Therefore, it is necessary to
50 construct such a new recombinant plasmid that the genes coding for β-D-galactosidase and β-galactoside permease derived from Escherichia coli lactose operon contained therein are able to be fully expressed in a strain of the genus Corynebacterium or Brevibacterium.

The present inventors have found that Escherichia coli lactose operon is expressed in a strain of the genus Corynebacterium or Brevibacterium sufficiently to confer the ability to assimilate lactose on the strain
55 by replacing the transcription and translation signals in Escherichia coli lactose operon by the transcription and translation signals derived from a strain of the genus Corynebacterium or Brevibacterium.

A recombinant plasmid which contains Escherichia coli lactose operon and allows such efficient gene expression as to confer the ability to assimilate lactose on a microorganism of the genus Corynebacterium

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or Brevibacterium can be prepared by inserting a DNA fragment, which is derived from a strain of the genus Corynebacterium or Brevibacterium and enables sufficient expression of Escherichia coli lactose operon in said strain, into a plasmid at a site upstream of the structural genes derived from Escherichia coli lactose operon. For example, such a plasmid can be constructed according to the following procedure:
5 isolating a DNA fragment containing the structural genes, lacZ and lacY, from the lactose operon of Escherichia coli KI2 strain; ligating this DNA fragment to a plasmid vector autonomously replicable in strains of the genus Corynebacterium or Brevibacterium ; further inserting a DNA fragment derived from a strain of the genus Corynebacterium or Brevibacterium and enabling expression of foreign genes at a site upstream of the coding region of lacZ and lacY; and selecting a recombinant plasmid which can confer the ability to
10 assimilate lactose on a strain of the genus Corynebacterium or Brevibacterium.

The DNA fragment containing a part or all of the coding region of the structural genes lacZ and lacY in lactose operon can be isolated by self-cloning of lactose operon according to the known in vitro recombination technique ("Molecular Cloning" by T. Maniatis. et al.; Cold Spring Harbor Laboratory. 1982) using the Escherichia coli host-vector system with a mutant derived from Escherichia coli KI2 strain unable
15 to assimilate lactose as a recipient, and with restoration of the ability to assimilate lactose, followed by subcloning of the structural genes, lacZ and lacY, by the use of suitable endonucleases and exonucleases. Alternatively, a DNA fragment which contains the structural genes lacZ and lacY already subcloned may be used; for example, plasmid pMCI871 which contains the structural gene lacZ and was prepared by Casadaban, et al. [Gene, 25, 71 (1983)] is preferably used. The plasmid pMCI871 is a vector for the detection
20 of the promoter activity of Escherichia coli. This plasmid contains a DNA fragment derived from Escherichia coli lactose operon, which lacks the promoter region and the region coding for eight N-terminal amino acids of β-D-galactosidase and contains the region encoding from the codon for the ninth amino acid to the EcoRI cleavage site located on the 5′-side of the codon for C-terminal amino acid. In addition, the coding region is preceded by multiple cloning site. In vitro recombination of pMCI871 at a site downstream of its lacZ-coding
25 region with a DNA fragment containing lacY-coding region from Escherichia coli lactose operon separately cloned gives a DNA fragment which does not contain the transcription and translation signals of Escherichia coli and contains structural gene lacY and a part of structural gene lacZ lacking a region coding for some N-terminal amino acids of β-D-galactosidase.

The DNA fragment containing the coding region of both lacZ and lacY thus obtained is then ligated to a
30 vector of a strain of the genus Corynebacterium or Brevibacterium .

Any vector autonomously replicable in a strain of the genus Corynebacterium or Brevibacterium may be used. Examples of such vectors include pCGI [Japanese Published Unexamined Patent Application No. 134500/82 (European Patent No. 58889, U.S. Patent No. 4617267)], pCG2 [Japanese Published Unexamined Patent Application No. 35197/83 (European Patent No. 73062, U.S. Patent No. 4489160)], pCG4 and pCGII
35 [Japanese Published Unexamined Patent Application No. 183799/82 (European Publication No. 63763, U.S. Patent No. 4500640)], pCE54 and pCBI0I [Japanese Published Unexamined Patent Application No. 105999/83 (European Publication No. 82485, U.S. Patent Application Serial No. 668674)], pCE5I, pCE52 and pCE53 [Mol. Gen. Genet., 196, 175 (1984)], and plasmids derived therefrom. These vector plasmid DNAs can be isolated from cultured cells of the strains containing the same and purified according to the methods
40 disclosed in Japanese Published Unexamined Patent Application No. 134500/82 (European Patent No. 58889, U.S. Patent No. 4617267) and Japanese Published Unexamined Patent Application No. 186489/82 (European Publication No. 64680, U.S. Patent No. 4681847).

The DNA fragment containing the structural genes lacZ and lacY cloned in the Escherichia coli host-vector system can be isolated by digestion with a restriction enzyme, followed by agarose gel elec-
45 trophoresis. Separately, the vector plasmid is cleaved with a restriction enzyme which gives cleaved terminals capable of being ligated with the above DNA fragment. The two elements are then ligated by treatment with T4 ligase, and the ligase reaction mixture is used to transform a strain of the genus Corynebacterium or Brevibacterium by the method using protoplasts [Japanese Published Unexamined Patent Application No. 186489/82 (European Publication No. 64680, U.S. Patent No. 4681847), Japanese
50 Published Unexamined Patent Application No. 186492/82 (European Publication No. 63764, U.S. Patent No. 4683205) and Japanese Published Unexamined Patent Application No. 126789/83 (European Publication No. 88166, U.S. Patent Application Serial No. 787010)]. Transformants are selected based on the selective marker of the vector. Plasmid can be obtained from the transformants, followed by analysis of their structure by digestion with restriction enzymes. The desired plasmid must have restriction enzyme cleavage
55 sites upstream of the coding region of lacZ and lacY in order to allow, in the subsequent step, the construction of a plasmid enabling the expression of the genes coding for β-D-galactosidase and β-galactoside permease. Plasmid pE'lacI disclosed in the Example of the present invention is prepared by ligating the lacZ-lacY DNA fragment obtained above to pCGII at its oligolinker site which is derived from

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MI3mpl9RF DNA, and hence has a desired structure wherein several restriction enzyme cleavage sites derived from oligolinker are positioned just before the lacZ-lacY structural genes.

A DNA fragment responsible for the initiation of transcription and translation in a strain of the genus Corynebacterium or Brevibacterium can be cloned by using said plasmid containing the structural genes lacZ and lacY and by detection of the expression of the gene as a detection marker: Any of the microorganisms known as coryneform bacteria belonging to the genus Corynebacterium or Brevibacterium may be used as the donor of the aforementioned DNA fragment and as the host microorganism for cloning. Preferred examples are the strains listed below and mutants derived therefrom.

Corynebacterium glutamicum   ATCC I3032
Corynebacterium glutamicum   ATCC 3I833
Corynebacterium acetoacidophilum   ATCC I3870
Corynebacterium herculis   ATCC I3868
Corynebacterium lilium   ATCC I5990
Brevibacterium divaricatum   ATCC I4020
Brevibacterium flavum   ATCC I4067
Brevibacterium immariophilium   ATCC I4068
Brevibacterium lactofermentum   ATCC I3869
Brevibacterium thiogenitalis   ATCC I9240

Cloning of the DNA fragment responsible for the initiation of transcription and translation in a strain of the genus Corynebacterium or Brevibacterium by the use of the above plasmid can be carried out in the following manner.

The plasmid is cleaved at the restriction enzyme cleavage site located just before the region coding for the structural genes lacZ and lacY. Separately, the chromosomal DNA of a strain of the genus Corynebacterium or Brevibacterium, which is the donor of a DNA fragment enabling the expression of a gene, is completely or partially digested with the same restriction enzyme as used above or another restriction enzyme which gives the same cohesive ends. The two digestion mixtures thus obtained are ligated by treatment with T4 ligase, and the ligase reaction mixture is used to transform the protoplast of a strain of the genus Corynebacterium or Brevibacterium. The protoplast suspension thus obtained is spread on a regeneration agar medium containing a vector marker reagent and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL).

Coryneform bacteria originally show no β-D-galactosidase activity, but the colonies of transformants which express this activity encoded by the structural gene lacZ show blue color on the selection medium. The recombinant plasmid having the DNA fragment responsible for the initiation of transcription and translation in coryneform bacteria, which is inserted upstream of the structural gene lacZ, can be isolated from cultured cells of the blue-colored transformants by a conventional method.

The development of blue color in colonies is caused by the hydrolysis of X-GAL with β-D-galactosidase, and therefore, a colony having a high β-D-galactosidase activity shows a deep blue color. Strains carrying the recombinant DNA which contains a DNA fragment responsible for the initiation of transcription and translation in coryneform bacteria to enable intense gene expression can be easily detected as deep blue colonies. The degree of gene expression in a blue-colored transformant can be correctly evaluated by subjecting cultured cells of the blue-colored transformant to ultrasonic disruption and measuring the β-D-galactosidase activity in the cell-free extract.

A shuttle vector for Escherichia coli and coryneform bacteria can be prepared by combining the plasmid obtained above which contains a DNA fragment carrying promoter region and ribosome-binding sequence derived from a coryneform bacterium with a vector autonomously replicable in Escherichia coli. The degree of gene expression of said promoter in Escherichia coli can be examined by introducing the shuttle vector into Escherichia coli. The degree of expression of β-D-galactosidase in Escherichia coli under the control of the promoter region and ribosome-binding sequence derived from a coryneform bacterium was determined by the method described below in Example (6). The result, as shown in Table 2, has revealed that a promoter-ribosome-binding sequence which allows an intense gene expression in a coryneform bacterium does not always show a high degree of expression in Escherichia coli. This fact indicates the difference between the region responsible for the initiation of transcription and translation in coryneform bacteria and that in Escherichia coli. It was thus confirmed that not only the promoter but also the region responsible for the initiation of translation must be derived from a coryneform bacterium in order to ensure full gene expression in coryneform bacteria.

The process established by the present invention to prepare the region responsible for the initiation of transcription and translation provides an effective means for obtaining the gene-expression control region for

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coryneform bacteria.

The DNA fragment carrying gene-expression controlling region and cloned on a plasmid as described above can be isolated by cleaving the plasmid with suitable restriction enzymes according to the cleavage map of that plasmid, followed by fractionation with agarose gel electrophoresis. The DNA fragment thus isolated is then inserted, by known recombinant DNA technology, into a plasmid at a site upstream of a desired structural gene to construct a recombinant DNA. By introducing the recombinant DNA into a coryneform bacterium, efficient synthesis of the desired gene product in the bacterium can be achieved. If the expression of a structural gene coding for an enzyme responsible for the biosynthesis of a metabolite such as an amino acid is intensified by this process, the productivity of the metabolite will be significantly increased. It is also possible to confer a new metabolic function which is not inherent in coryneform bacteria such as the ability to assimilate lactose, or to give an ability to produce physiologically active proteins derived from eukaryotes by expression of a structural gene derived from foreign microorganisms or eukaryotes.

Plasmid pE'lacI, which enables cloning of the DNA fragment responsible for the transcription and translation in coryneform bacteria, was introduced into Corynebacterium glutamicum ATCC 31833, and the transformant thus obtained was deposited with the Fermentation Research Institute (FRI), Agency of Industrial Science and Technology, Japan on September 27, 1986 as Corynebacterium glutamicum K73 (FERM BP-1178).

A recombinant DNA which can confer the ability to assimilate lactose on a strain of the genus Corynebacterium or Brevibacterium is derived from the plasmid autonomously replicable in a strain of the genus Corynebacterium or Brevibacterium and containing a DNA fragment which carries the structural genes lacZ and lacY in the following manner. That is, the said plasmid is cleaved with a restriction enzyme at the cleavage site located just before the structural genes. Separately, in order to obtain a DNA fragment enabling expression of foreign genes, the chromosomal DNA of a strain of the genus Corynebacterium or Brevibacterium is completely or partially digested with the same restriction enzyme as used above or another restriction enzyme which gives the same cleaved terminals. The two digestion mixtures thus obtained are ligated by treatment with T4 ligase, and the ligase reaction mixture is used to transform the protoplast of a strain of the genus Corynebacterium or Brevibacterium. The protoplast suspension thus obtained is spread on regeneration agar medium containing a vector marker reagent and X-GAL which enables detection of a clone having β-D-galactosidase activity. Transformants which have acquired the ability to assimilate lactose can be isolated by selecting blue-colored colonies grown on the selection agar medium, and a strain which can grow using lactose as a single carbon source can be selected from these transformants. A strain of the genus Corynebacterium or Brevibacterium becomes capable of assimilating lactose by transformation with the plasmid isolated from the transformant obtained above.

This fact indicates that the ability to assimilate lactose is conferred by said plasmid. Thus, a recombinant plasmid which can confer the ability to assimilate lactose on a strain of the genus Corynebacterium or Brevibacterium can be constructed. One example of such a recombinant plasmid is pCPL7 derived from pE'lacI, in which a DNA fragment of 3.1 kb obtained by cleavage of the chromosomal DNA of a strain of the genus Corynebacterium with restriction enzyme Sau3A has been inserted at a site upstream of the structural gene lacZ of pE'lacI to enable expression of foreign genes.

The recombinant plasmid which can confer the ability to assimilate lactose thus prepared can be introduced into various amino acid-producing strains of the genus Corynebacterium or Brevibacterium by the above-described method using protoplasts.

Examples of the microorganisms into which this recombinant plasmid is to be introduced include the strains known as glutamic acid-producing coryneform bacteria such as Corynebacterium glutamicum, Corynebacterium herculis, Brevibacterium flavum and Brevibacterium lactofermentum. Transformants actually obtained are shown in the Example described later. A typical example is Corynebacterium glutamicum K74, which is a transformant obtained by introducing plasmid pCPL7 into Corynebacterium glutamicum ATCC 31833. This strain was deposited with FRI on September 27, 1986 as Corynebacterium glutamicum K74 (FERM BP-1179).

The coryneform bacterium capable of assimilating lactose thus prepared takes in lactose through the cell membrane by the action of β-galactoside permease, hydrolyzes it into galactose and glucose by the action of β-D-galactosidase, and produces an amino acid by utilizing the glucose formed.

Production of amino acids by the strains carrying such a recombinant plasmid may be carried out by the same culture method as in the case where glucose or molasses is used as a carbon source. That is, the transformant is cultured under aerobic conditions in a medium containing, as a carbon source, a lactose-containing substance in place of glucose or molasses, and other nutrients such as nitrogen sources, inorganic compounds, amino acids and vitamins at a controlled temperature and pH, whereby a desired L-

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amino acid can be produced in the culture broth. In general, loss of plasmid is likely to occur during the culturing of plasmid-carrying strains. In the process of the present invention, strains which have lost the recombinant plasmid fail to grow in the medium containing a lactose-containing substance as the main carbon source, and consequently, the recombinant plasmid is stably retained.

5   Lactose-containing substances such as cheese whey, casein whey, and products obtained therefrom by extraction or concentration can be used as a carbon source, as well as lactose itself.

    Further, as the carbon source, carbohydrates such as glucose, glycerol, fructose, sucrose, maltose, mannose, starch, starch hydrolyzate, molasses, etc.; polyalcohols; and various organic acids such as pyruvic acid, fumaric acid, lactic acid, acetic acid, etc. can be used. Furthermore, hydrocarbons, alcohols,
10 etc. can be used, depending upon the assimilability of the microorganism to be used.

    As the nitrogen source, ammonia; inorganic or organic ammonium salts such as ammonium chloride, ammonium sulfate, ammonium carbonate and ammonium acetate; urea and other nitrogen-containing inorganic substances; and nitrogen-containing organic substances such as peptone, NZ-amine, meat extract, yeast extract, corn steep liquor, casein hydrolyzate, fish meal or its digested product and chrysalis
15 hydrolyzate may be used.

    As the inorganic compound, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ammonium sulfate, ammonium chloride, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate, etc. may be used. If vitamins, amino acids, etc. required for the growth of the microorganism are supplied by other medium components described above, it is not necessary to add
20 these specific nutrients separately to the medium.

    Culturing is carried out under aerobic conditions by shaking culture, aeration-stirring culture, etc. The preferred culturing temperature is generally 20-40°C. It is desirable that the pH of the culture medium is maintained around neutrality. L-amino acids is usually accumulated in the culture medium after culturing for 1-5 days under these conditions. After completion of the culturing, the cells are removed from the culture
25 broth, and L-amino acid is recovered from the supernatant by a known method established for the particular amino acid.

    For the production of L-glutamic acid using, as a carbon source, molasses which contains biotin at a high concentration, various methods have been proposed: a method in which an antibiotic such as penicillin is added (Japanese Published Examined Patent Application No. 1695/62), a method in which a surface-
30 active agent or a saturated fatty acid is added (Japanese Published Examined Patent Application Nos. 8798/65 and 14559/65), a method in which an oleic acid-requiring strain is used (Japanese Published Examined Patent Application No. 19632/75) and a method in which a glycerol-requiring strain is used. (Japanese Published Examined Patent Application No. 33997/76). Since whey also generally contains biotin at a high concentration, the above methods may be employed advantageously in the production of L-
35 glutamic acid using whey as a carbon source.

    Thus, lactose-containing substances can be used as the main carbon source for the production of various amino acids such as L-glutamic acid, L-glutamine, L-lysine, L-threonine, L-isoleucine, L-valine, L-leucine, L-tryptophan, L-phenylalanine, L-tyrosine, L-histidine, L-arginine, L-ornithine, L-citrulline and L-proline by amino acid-producing strains of the genus Corynebacterium or Brevibacterium carrying a
40 recombinant plasmid which can confer the ability to assimilate lactose.

    Certain specific embodiments of the present invention are illustrated by the following representative example.

45 Example

(I) Cloning of Escherichia coli lactose operon

    Chromosomal DNA was isolated from cultured cells of Escherichia coli K294 (FERM BP-526), which
50 was derived from Escherichia coli K12 and having the ability to assimilate lactose, according to the method of Marmur, et al. [J. Mol. Biol., 3, 208 (1961)].

    pBR322 [Gene, 2, 95 (1975)] commercially available from Takara Shuzo Co., Ltd. was used as a cloning vector.

    Four units of restriction enzyme Bgl II (a product of Takara Shuzo Co., Ltd.) was added to 90 μl of
55 reaction buffer solution for BglII [10 mM tris(hydroxymethyl)aminomethane (hereinafter referred to as Tris), 10 mM MgCl₂, 100 mM NaCl and 1 mM dithiothreitol, pH 7.5] containing 2 μg of the chromosomal DNA prepared above. The mixture was subjected to reaction at 37°C for 60 minutes. Then, the reaction was stopped by heating the reaction mixture at 65°C for 10 minutes. Separately, 2 units of restriction enzyme

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Bam HI (a product of Takara Shuzo Co., Ltd.) was added to 90 μl of reaction buffer solution for BamHI (l0 mM Tris, 10 mM MgCl₂, 100 mM NaCl and 1 mM dithiothreitol, pH 7.5) containing 1 μg of pBR322 DNA. The mixture was subjected to reaction at 37°C for 60 minutes. Then, the reaction was stopped by heating the reaction mixture at 65°C for 10 minutes.

Both reaction mixtures were mixed, and then 20 μl of buffer solution for T4 ligase at a 10-fold concentration (660 mM Tris, 66 mM MgCl₂ and 100 mM dithiothreitol, pH 7.6), 2 μl of 100 mM ATP and 350 units of T4 ligase (a product of Takara Shuzo Co., Ltd.) were added thereto. The mixture was subjected to ligase reaction at 12°C for 16 hours. The reaction mixture was used for the transformation of Escherichia coli K72 (lac , leu), which was derived from Escherichia coli K12 and deposited with FRI as Esherichia coli K72 FERM BP-1176 on September 27, 1986.

The transformation was carried out according to the method of Dagert, et al. [Gene. 6, 23 (1979)]. E-scherichia coli K72 strain was inoculated into 50 ml of L-medium (10 g·l Bacto-tryptone, 5 g·l yeast extract, 1 g/l glucose and 5 g/l NaCl, pH 7.2), and cultured at 37°C until the optical density (OD) measured at 660 nm with a Tokyo Koden colorimeter reached 0.5 (hereinafter OD is measured at 660 nm unless otherwise specified). The culture broth was cooled on ice for 10 minutes and centrifuged. The cells collected were suspended in 20 ml of cooled 0.1 M CaCl₂ and the suspension was left standing at 0°C for 20 minutes. After centrifugation of the suspension, the collected cells were resuspended in 0.5 ml of 0.1 M CaCl₂ and the suspension was left standing at 0°C for 18 hours. To this suspension of CaCl₂-treated cells (150 μl), was added 50 μl of the ligase reaction mixture obtained above. The resulting mixture was left standing at 0°C for 10 minutes and then heated at 37°C for 5 minutes. L-medium (2 ml) was further added thereto and shaking culture was carried out at 37°C for 2 hours.

The culture broth thus obtained was spread on L-agar-medium (L-medium containing 16 g/l agar) containing 100 μg/ml ampicillin and 40 μg/ml X-GAL, and incubation was carried out at 37°C for one day. The blue-colored colonies grown on the agar medium were collected, and their properties were examined. It was found that this strain could grow on a minimal medium containing lactose as a carbon source and leucine (10 g/l lactose, 7 g/l K₂HPO₄, 2 g/l KH₂PO₄, 0.5 g/l disodium citrate dihydrate, 1 g/l (NH₄)₂SO₄, 0.1 g/l MgSO₄•7H₂O, 0.1 mg/l thiamine hydrochloride, 50 mg/l leucine and 16 g/l agar, pH 7.2) but could not grow on L-agar-medium containing 25 μg/ml tetracycline.

From this transformant, a plasmid DNA was isolated according to the method of Ann, et al. [J. Bacteriol., 140, 400 (1979)]. The plasmid DNA was digested with restriction enzymes, and analyzed by agarose gel electrophoresis. It was found that the plasmid had a size of 15.3 kilobase (kb), and a structure wherein a BglII-cleaved DNA fragment of 11 kb derived from the chromosomal DNA of Escherichia coli K294 strain was inserted in pBR322 at the BamHI-cleavage site. This plasmid was named pLacI.

(2) Construction of novel vector plasmid pCGIII

A novel vector pCGIII was constructed by ligating vector pCGI [Japanese Published Unexamined Patent Application No. 183799/82 (European Publication No. 63763, U.S. Patent No. 4500640)] autonomously replicable in Corynebacterium glutamicum with a multilinker contained in MI3mpI9RF DNA according to the procedure described below.

pCGII was isolated from cultured cells of Corynebacterium glutamicum ATCC 39819 (a lysozyme-sensitive mutant derived from Corynebacterium glutamicum ATCC 31833) carrying pCGII in the following manner.

The above mutant was cultured with shaking at 30°C in 400 ml of NB medium (20 g/l bouillon powder and 5 g/l yeast extract, pH 7.2) until OD reached about 0.7. The cultured cells were collected, washed with TES buffer solution [0.03 M Tris, 0.005 M disodium ethylenediaminetetraacetate (EDTA) and 0.05 M NaCl, pH 8.0], and suspended in 10 ml of a lysozyme solution (25% sucrose, 0.1 M NaCl, 0.05 M Tris and 0.8 mg/ml lysozyme, pH 8.0). The suspension was subjected to reaction at 37°C for 2 hours. Then, 2.4 ml of 5 M NaCl, 0.6 ml of 0.5 M EDTA (pH 8.5) and 4.4 ml of a solution comprising 4% sodium laurylsulfate and 0.7 M NaCl were successively added to the reaction mixture, and the mixture was gently stirred and placed on ice for 15 hours. The mixture was then transferred to a centrifuge tube and centrifuged at 69,000 × g at 4°C for 60 minutes to recover a supernatant. Then, polyethylene glycol (PEG) 6000 (a product of Nakarai Chemicals, Ltd.) was added thereto in an amount corresponding to 10% by weight. The mixture was gently stirred, and then placed on ice. After 10 hours, the mixture was centrifuged at 1,500 × g for 10 minutes to recover pellets.

Then, 5 ml of TES buffer solution was added to gradually dissolve the pellets, and 2.0 ml of 1.5 mg/ml ethidium bromide was added to the solution. Cesium chloride was further added to adjust the

density of the solution to 1.580.

The solution thus obtained was subjected to ultracentrifugation at 105,000 $\times$ g at 18°C for 48 hours, and a high density band at the lower position of the centrifuge tube detected under ultraviolet irradiation was withdrawn by puncturing the side of the centrifuge tube, using a 21-gauge needle to recover pCGII plasmid

5 DNA. The fraction was treated five times with an equal volume of isopropyl alcohol solution [90% (V V) isopropyl alcohol in TES buffer solution] containing a saturated amount of cesium chloride to remove ethidium bromide by extraction. Then, the solution was dialyzed against TES buffer solution.

To 90 $\mu\ell$ of reaction buffer solution for restriction enzyme SstI (10 mM Tris, 10 mM MgCl2 and 1 mM dithiothreitol, pH 7.5) containing 1 μg of pCGII plasmid DNA obtained above, was added 1 unit of SstI (a

10 product of Takara Shuzo Co., Ltd.), and the mixture was subjected to reaction at 37°C for 60 minutes. Then, 10 $\mu\ell$ of 1 M NaCl and 1 unit of PstI (a product of Takara Shuzo Co., Ltd.) were added thereto, and the reaction was continued at 37°C for an additional 60 minutes and then stopped by heating the reaction mixture at 65°C for 10 minutes.

Similarly, 1 μg of MI3mpl9RF DNA (a product of Takara Shuzo Co., Ltd.) was digested with SstI and

15 PstI, and the reaction was stopped by heating. Both reaction mixtures were mixed, and then 20 $\mu\ell$ of buffer solution for T4 ligase at a 10-fold concentration, 2 $\mu\ell$ of 100 mM ATP and 350 units of T4 ligase were added thereto. The mixture was subjected to ligase reaction at 12°C for 16 hours. The reaction mixture was used for the transformation of Corynebacterium glutamicum ATCC 31833.

The transformation was carried out by using the protoplasts prepared in the following manner. A seed

20 culture of Corynebacterium glutamicum ATCC 31833 cultured in NB medium was inoculated into semi-synthetic medium SSM [20 g/ℓ glucose, 10 g/ℓ (NH$_4$)$_2$SO$_4$, 3 g/ℓ urea, 1 g/ℓ yeast extract, 1 g/ℓ KH$_2$PO$_4$, 0.4 g/ℓ MgCl$_2$•6H$_2$O, 10 mg/ℓ FeSO$_4$•7H$_2$O, 0.2 mg/ℓ MnSO$_4$•4-6H$_2$O, 0.9 mg/ℓ ZnSO$_4$•7H$_2$O, 0.4 mg/ℓ CuSO$_4$•5H$_2$O, 0.09 mg/ℓ Na$_2$B$_4$O$_7$•10H$_2$O, 0.04 mg/ℓ (NH$_4$)$_6$Mo$_7$O$_{24}$•4H$_2$O, 30 μg/ℓ biotin and 1 mg/ℓ thiamine hydrochloride, pH 7.2 (hereinafter referred to as SSM medium)], and cultured with shaking at 30°C. When

25 OD reached 0.2, penicillin G was added to the culture medium to a concentration of 0.5 unit/mℓ, and culturing was continued until OD reached 0.6.

The cells were collected from the culture broth, and suspended to a concentration of about 10^9 cells per milliliter in RCGP medium [5 g/ℓ glucose, 5 g/ℓ Casamino acid, 2.5 g/ℓ yeast extract, 3.5 g/ℓ K$_2$HPO$_4$, 1.5 g/ℓ KH$_2$PO$_4$, 0.41 g/ℓ MgCl$_2$•6H$_2$O, 10 mg/ℓ FeSO$_4$•7H$_2$O, 2 mg/ℓ MnSO$_4$•4-6H$_2$O, 0.9 mg/ℓ ZnSO$_4$•7H$_2$O,

30 0.04 mg/ℓ (NH$_4$)$_6$Mo$_7$O$_{24}$•4H$_2$O, 30 μg/ℓ biotin, 2 mg/ℓ thiamine hydrochloride, 135 g/ℓ disodium succinate and 30 g/ℓ polyvinyl pyrrolidone having an average molecular weight of 10,000, pH 7.6] containing 1 mg/mℓ lysozyme. The suspension was transferred into an L-type test tube and gently shaken at 30°C for 5 hours to prepare protoplasts.

Then, 0.5 mℓ of the protoplast suspension was taken in a small test tube and centrifuged at 2,500 $\times$ g

35 for 5 minutes to separate the protoplasts. The protoplasts were re-suspended in 1 mℓ of TSMC buffer solution (10 mM MgCl$_2$, 30 mM CaCl$_2$, 50 mM Tris and 400 mM sucrose, pH 7.5) and washed by centrifugation. The protoplasts were resuspended in 0.1 mℓ of TSMC buffer solution. Then, 100 $\mu\ell$ of a 1:1 mixture of TSMC buffer solution at a two-fold concentration and the ligase reaction mixture obtained above was added to the protoplast suspension, and the resulting mixture was further admixed with 0.8 mℓ of TSMC buffer solution

40 containing 20% PEG 6000. After three minutes, 2 mℓ of RCGP medium (pH 7.2) was added thereto, and the resulting mixture was centrifuged at 2,500 $\times$ g for 5 minutes to remove a supernatant. The precipitated protoplasts were suspended in 1 mℓ of RCGP medium, and 0.2 mℓ of the suspension was spread on RCGP agar medium (RCGP medium containing 1.4% agar, pH 7.2) containing 400 μg/mℓ spectinomycin and cultured at 30°C for 10 days.

45 Several colonies grown on RCGP agar medium were picked up, and plasmid DNAs were isolated from them as follows. Each of the colonies was inoculated into SSM medium, and cultured with shaking at 30°C until OD reached 0.2. Penicillin G was added to the culture medium to a concentration of 0.5 unit/mℓ, and culturing was further continued until OD reached 0.6. Plasmid DNAs were isolated from the cultured cells by the alkali method (Molecular Cloning, p.368).

50 Digestion with restriction enzymes and analysis by agarose gel electrophoresis revealed that one of the plasmid DNAs thus isolated was composed of a partially digested product (PstI-Sst I) of pCGII and PstI-SstI linker of MI3mpl9RF DNA (refer to Figure I). The plasmid was named pCGIII. This plasmid is a very useful plasmid because it has KpnI, SmaI, BamHI, XbaI, SalI and PstI cleavage sites derived from MI3mpl9 multilinker and is digested only at one site by each of these restriction enzymes.

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(3) Preparation of vector plasmid pE'lacl for the cloning of the region responsible for the initiation of transcription and translation in coryneform bacteria

Vector plasmed pE'lacl for the cloning of the region responsible for the initiation of transcription and
5 translation in coryneform bacteria was constructed by the following method using plasmid pLacl containing Escherichia coli lactose operon cloned in (I) above, vector plasmid pCGIII prepared in (2) above and pMCl871 (refer to Figure 2).

pMCl871 commercially available froa Pharmacia Fine Chemicals was used in this experiment. Four units of restriction enzyme EcoRl (a product of Takara Shuzo Co., Ltd.) was added to 50 μℓ of reaction buffer
10 solution for EcoRl (10 mM Tris, 10 mM MgCl₂, 100 mM NaCl and 1 mM dithiothreitol, pH 7.5) containing 2 μg of pMCl871 plasmid DA. The mixture was subjected to reaction at 37°C for 60 minutes. The reaction mixture was subjected to agarose gel electrophoresis, and about 0.4 μg of an EcoRl-cleaved DNA fragment of 3.0 kb was obtained by the method of Girvitz, et al. (Molecular Cloning p.I68).

Separately, 8 units of EcoRl was aided to 50 μℓ of reaction buffer solution for EcoRl containing 4 μg of
15 pLacl plasmid DNA prepared in (I) above, and the mixture was subjected to reaction at 37°C for 60 minutes. Then, 5 μℓ of 1 M NaCl and 8 units of restriction enzyme Sall (a product of Takara Shuzo Co., Ltd.) were added thereto, and the reaction was continued at 37°C for an additional 60 minutes. About 0.4 μg of an EcoRl-Sall-cleaved DNA fragment of 3.2 kb was obtained from the reaction mixture by the same method as described above. It is evident from the cleavage map of lactose operon [Experiments with Gene Fusions by
20 T.J. Silhavy, et al.; Cold Spring Harbor Laboratory (1984)] that this DNA fragment contains the structural gene lacY of Escherichia coli .

Each of the two DNA fragments obtained above was dissolved in 45 μℓ of TES buffer solution, and the two solutions were mixed. Then, 10 μℓ of buffer solution for T4 ligase at a 10-fold concentration, 1 μℓ of 100 mM ATP and 350 units of T4 ligase were added thereto, and the mixture was subjected to ligase reaction at
25 12°C for 16 hours. Further, 10 μℓ of 1 M NaCl and 10 units of BamHI were added thereto, and the mixture was subjected to reaction at 37°C for 60 minutes. The reaction was stopped by heating the reaction mixture at 65°C for 10 minutes.

Separately, shuttle vector pCE53 autonomously replicable in both Escherichia coli and Corynebacterium glutamicum [Mol. Gen. Genet., 196, 175 (1984)] was isolated from Escherichia coli.KI2 strain carrying pCE53
30 by the method of Ann, et al. To 60 μℓ of reaction buffer solution for BamHI containing 1 μg of pCE53 plasmid DNA, was added 2 units of BamHI, and the mixture was subjected ot reaction at 37°C for 60 minutes. Then, 6 μℓ of 1 M Nacl and 2 units of Sall were added thereto, and the reaction was continued at 37°C for 60 minutes and then stopped by heating the reaction mixture at 65°C for 10 minutes.

Both reaction mixtures obtained above were mixed. Then, 20 μℓ of buffer solution for T4 ligase at a 10-
35 fold concentration, 2 μℓ of 100 mM ATP and 350 units of T4 ligase were added thereto, and the mixture was subjected to ligase reaction at 12°C for 16 hours. The ligase reaction mixture thus obtained was used for the transformation of Escherichia coli K294 strain according to the method of Dagert, et al., using L-agar medium containing 20 μg/mℓ kanamycin as a selection medium. From the kanamycin-resistant strains thus obtained, were selected the strains which did not grow on L-agar medium containing 25 μg/mℓ tetracycline.
40 Plasmids were isolated from these kanamycin-resistant and tetracycline-sensitive strains by the alkali method, and were digested with restriction enzymes, followed by analysis by agarose gel electrophoresis. It was found that one of these plasmids was a plasmid DNA having a size of 16.8 kb as shown in Figure 2, which was named pE'lac53l.

Corynebacterium glutamicum ATCC 31333 was transformed with 1 μg of pE'lac53l prepared above in the
45 same manner as in (2). RCGP agar medium containing 200 μg/mℓ kanamycin was employed as a selection medium, and a plasmid DNA was isolated from the transformant by the alkali method. The plasmid thus isolated was confirmed to be the same plasmid as pE'lac53l by digestion analysis with restriction enzymes. The plasmid pE'lac53l thus obtained (1 μg) and 1 μg of pCGIII prepared in (2) above were dissolved in 80 μℓ of reaction buffer solution for BamHI. Four units of BamHI was added thereto, and the mixture was
50 subjected to reaction at 37°C for 60 minutes. Then, 10 μℓ of 1 M NaCl and 4 units of Sall were added thereto, and the reaction was continued at 37°C for 60 minutes and then stopped by heating at 65°C for 10 minutes. 10 μℓ of buffer solution for T4 ligase at a 10-fold concentration, 1 μℓ of 100 mM ATP and 350 units of T4 ligase were further added, and the mixture was subjected to ligase reaction at 12°C for 16 hours. The ligase reaction mixture thus obtained was used for the transformation of Corynebacterium glutamicum
55 ATCC 31833 in the same manner as in (2) above, using RCGP agar medium containing 400 μg/mℓ spectinomycin as a selection medium.

Plasmids were isolated from the thus obtained spectinomycin-resistant transformants by the alkali method. The plasmids thus obtained were digested with restriction enzymes, and analyzed by agarose gel

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electrophoresis. It was found that one of the transformants carried a plasmid having a size of II kb as shown in Figure 2. The plasmid, named pE'lacI, carries the replication origin and pectinomycin-resistance gene obtained from plasmids pCGI and pCG4, respectively, the structural genes for Escherichia coli lactose operon in which promoter and eight amino acids on N-terminal side of lacZ are deleted, and BamHI, SmaI and KpnI cleavage sites positioned upstream of said structural genes.

(4) Cloning of a DNA fragment responsible for the initiation of transcription and translation in coryneform bacteria and construction of a recombinant plasmid which confers the ability to assimilate lactose on coryneform bacteria

A DNA fragment responsible for the initiation of transcription and translation derived from the chromosomal DNA of Corynebacterium glutamicum ATCC 31833 was cloned by using pE'lacI prepared in (3) above.

The chromosomal DNA of Corynebacterium glutamicum ATCC 31833 was prepared in the following manner.

A seed culture of Corynebacterium glutamicum ATCC 31833 cultured in NB medium was inoculated into 400 mℓ of SSM medium, and shaking culture was carried out at 30°C. When OD reached 0.2, penicillin G was added to the culture medium to a concentration of 0.5 unit/mℓ, and culturing was continued until OD reached 0.6.

The cells collected from the culture broth were washed with TES buffer solution, suspended in I0 mℓ of a lysozyme solution, and subjected to reaction at 37°C for 4 hours. Then, chromosomal DNA was isolated from the collected cells by the method of Saito and Miura [Biochim. Biophys. Acta, 72, 6I9 (I963)].

The chromosomal DNA thus obtained was partially digested with restriction enzyme Sau3A. That is, to 90 μℓ of reaction buffer solution for Sau3A (50 mM Tris, I0 mM MgCl₂, I00 mM NaCl and I mM dithiothreitol, pH 7.5) containing 2 μg of the chromosomal DNA, was added 0.5 unit of Sau3A (a product of Takara Shuzo Co., Ltd.). The mixture was subjected to reaction at 37°C for 30 minutes, and the reaction was stopped by heating at 65°C for I0 minutes.

Separately, 2 units of restriction enzyme BamHI was added to 90 μℓ of reaction buffer solution for BamHI containing I μg of vector pE'lacI DNA. The mixture was subjected to reaction at 37°C for 60 minutes to completely digest the plasmid, and the reaction was sto>ped by heating at 65°C for I0 minutes. Both reaction mixtures obtained above were mixed, and 20 μℓ of buffer solution for T4 ligase at a I0-fold concentration, 2 μℓ of I00 mM ATP ani 350 units of T4 ligase were added thereto. The mixture was subjected to ligase reaction at I2°C for I6 hours. The reaction mixture thus obtained was used for the transformation of Corynebacterium glutamicum ATCC 31833 in the same manner as in (2) above, using RCGP agar medium containing 400 μg/mℓ spectinomycin and 40 μg/mℓ X-GAL as a selection medium. After culturing at 30°C for 7 days, several blue-colored colonies were collected, and plasmids were isolated from these strains by the alkali method. Digestion with restriction enzymes and analysis by agarose gel electrophoresis revealed that the DNA fragment derived from the chromosomal DNA of Corynebacterium glutamicum ATCC 31833 was inserted into pE'lacI at the BamHI cleavage site. These plasmids were named pCPLI to pCPLI5. Of these, pCPL7 was found to be a plasmid containing a DNA fragment of 3.I kb. The strain carrying pCPL7 has been deposited as Corynebacterium glutamicum K74 (FERM BP-II79).

(5) Evaluation of cloned promoter

The degree of β-D-galactosidase expression in Corynebacterium glutamicum ATCC 31833 strains carrying plasmids pCPLI to pCPLI5 respectively obtained in (4) above was examined in the following manner. The cells of each strain cultured in 40 mℓ of NB medium containing I00 μg/mℓ spectinomycin were collected, suspended in 50 mM Tris-HCl buffer solution (pH 7.0), and disrupted by ultrasonic treatment under ice cooling for 20 minutes. The disrupted cell suspension was centrifuged to recover a cell extract as the supernatant, which was used to measure the activity of intracellular β-D-galactosidase and the β-D-galactosidase content in intracellular proteins. The β-D-galactosidase activity was determined by the method using o-nitrophenyl-β-D-galactoside (ONPG) [J.H. Miller, "Experiments in Molecular Genetics", p.403 (I972)]. The protein content of the cell extract was measured by using Bio-Rad's assay kit. Table I shows the specific activity of intracellular β-D-galactosidase in Corynebacterium glutamicum ATCC 31833 strains carrying plasmids pCPLI to pCPLI5 and vector pE'lacI respectively.

At the same time, the pattern of intracellular proteins in these strains was investigated through SDS-

polyacrylamide gel electrophoresis [Laemmli. Nature. 227. 680 (l980)]. A protein band which was not found with the strain carrying vector pE'lacl was observed with the strains carrying pCPLI to pCPLI5. The positions of these bands were nearly the same as that of β-D-galactosidase and intensity of the protein bands corresponded to the level of the intracellular β-D-galactosidase activities shown in Table I.

5   The results obtained above clearly show that DNA fragments containing the region responsible for the initiation of transcription and translation derived from a strain of the genus <u>Corynebacterium</u> or <u>Brevibacterium</u> can be prepared by using vector pE'lacl of the present invention.

Table 1

Strain	Specific Activity (U/mg)
<u>Corynebacterium</u> <u>glutamicum</u> ATCC 31833/pCPL1	2200
" ATCC 31833/pCPL2	2100
" ATCC 31833/pCPL3	2900
" ATCC 31833/pCPL4	3200
" ATCC 31833/pCPL5	880
" ATCC 31833/pCPL6	1000
" ATCC 31833/pCPL7	3000
" ATCC 31833/pCPL8	1100
" ATCC 31833/pCPL9	76
" ATCC 31833/pCPL10	2500
" ATCC 31833/pCPL11	7600
" ATCC 31833/pCPL12	1400
" ATCC 31833/pCPL13	1100
" ATCC 31833/pCPL14	550
" ATCC 31833/pCPL15	81
" ATCC 31833/pE'lacl	<1

45 (6) Gene expression in <u>Escherichia coli</u> by using the region responsible for the initiation of transcription and translation derived from a strain of the genus <u>Corynebacterium</u> or <u>Brevibacterium</u>

Shuttle plasmids for <u>Escherichia coli</u> and coryneform bacteria were constructed by inserting plasmid vector pACYCI77 [Chang, <u>et al.</u>, J. Bacteriol, I34, II4I (I978)], which is autonomously replicable in <u>Escherichia</u>
50 <u>coli</u> and has kanamycin-and ampicillin-resistance markers, into pE'lacl and each of the plasmids pCPL3, I0, I2 and I4 obtained in (4) above at the <u>Pstl</u> cleavage site. The shuttle plasmids were introduced into <u>E-scherichia coli</u> to examine the degree of expression of the gene coding for β-D-galactosidase therein.

One unit of restriction enzyme <u>Pstl</u> (a product of Takara Shuzo Co., Ltd.) was added to 45 μl of reaction buffer solutions for <u>Pstl</u> (I0 mM Tris, I0 mM MgCl₂, I00 mM NaCl and I mM dithiothreitol, pH 7.5)
55 containing 0.5 μg of pE'lacl, pCPL3, pCPLI0, pCPLI2 and pCPLI4, respectively. The mixture was subjected to reaction at 37°C for 60 minutes, and the reaction was stopped by heating at 65°C for I0 minutes.

Separately, 6 units of <u>Pstl</u> was added to 225 μl of reaction buffer solution for <u>Pstl</u> containing 2.5 μg of plasmid pACYCI77, and the reaction was allowed to proceed and stopped in the same manner as above.

Forty-five μl each of both reaction mixtures obtained above were mixed. and 10 μl of buffer solution for T4 ligase at a 10-fold concentration. 1 μl of 100 mM ATP and 350 units of T4 ligase were added thereto. The mixture was subjected to ligase reaction at 12°C for 16 hours. The ligase reaction mixture thus obtained was used for the transformation of Escherichia coli K72 derived from Escherichia coli K12 strain. in the same manner as in (1) above. using L-agar medium containing 100 $\mu g.ml$ spectinomycin and 20 $\mu g ml$ kanamycin as a selection medium. The colonies grown on the agar medium were picked up, and plasmids were isolated from the transformants by the method of Ann, et al. The plasmid DNAs thus obtained were digested with restriction enzymes, and analyzed by agarose gel electrophoresis. It was confirmed that the plasmids had structures wherein pACYC177 DNA fragment of 3.6 kb was inserted into pE'lacI and plasmids pCPL3, 10, 12 and 14, respectively. at the PstI cleavage site. These plasmids were named pCPLI03. II0, II2. II4 and pE'lacI0I, respectively. Each of these plasmids was used for the transformation of Corynebacterium glutamicum ATCC 31833 in the same manner as in (2) above to obtain a spectinomycin-and kanamycin-resistant transformant.

Corynebacterium glutamicum ATCC 31833 and Escherichia coli K72 strains carrying pE'lacI0I and pCPLI03, II0, II2 and II4 obtained above, respectively, were examined for the specific activity of intracellular β-D-galactosidase by the same method as in (5) above. except that the ultrasonic treatment was carried out for 2 minutes for the transformants of K72 strain. The results are shown in Table 2.

Table 2

Strain	Specific Activity (U/mg)
Corynebacterium glutamicum ATCC 31833/pCPL103	10,000
" ATCC 31833/pCPL110	440
" ATCC 31833/pCPL112	1,500
" ATCC 31833/pCPL114	570
" ATCC 31833/pE'lacl01	<1
Escherichia coli K72/pCPL103	9
" K72/pCPL110	30
" K72/pCPL112	11
" K72/pCPL114	21
" K72/pE'lacl01	<1

As shown in Table 2. gene expression using the region responsible for the initiation of transcription and translation derived from Corynebacterium glutamicum in Escherichia coli is feeble and. has no correlation with that in Corynebacterium glutamicum . This fact indicates that there is a difference in nature between the region responsible for the initiation of transcription and translation in strains of the genus Corynebacterium or Brevibacterium and the promoter and ribosome-binding sequence in Escherichia coli.

(7) Conferment of the ability to assimilate lactose on strains of the genus Corynebacterium or Brevbacterium by introduction of pCPL7

Corynebacterium glutamicum ATCC 31833, Corynebacterium herculis ATCC 13868. Brevibacterium flavum ATCC 14067 and Brevibacterium lactofermentum ATCC 13869 were transformed by using 1 μg of pCPL7 according to the same method as in (4) above. It was confirmed that plasmids isolated from the spectinomycin-resistant transformants which formed blue-colored colonies were identical with pCPL7.

A culture test of the transformants obtained above and their parent strains in a medium containing lactose as a carbon source was carried out in the following manner. Each strain was cultured with shaking in NB medium at 30°C for 24 hours. For the culture of the transformants. spectinomycin was added to NB medium at a concentration of I00 μg.ml. Then, the cells recovered from 0.5 ml of the seed culture broth
5 were washed with physiological saline solution by centrifugation and then inoculated into I0 ml of a minimal medium containing lactose as a carbon source [I0 g/l lactose, I0 g/l (NH₄)₂SO₄, I g/l KH₂PO₄, 0.4 g/l MgSO₄•7H₂O, 2 mg/l FeSO₄•7H₂O, 2 mg/l MnSO₄•4H₂O, 60 μg/l biotin, 2 mg/l thiamine hydrochloride, 3 g/l urea and 50 mg/l NaCl. pH 7.2] in an L-type test tube. Culturing was carried out with shaking at 30°C for I2 hours, and turbidity at 660 nm was measured. The results are shown in Table 3.
10

Table 3

Strain		ΔOD_{660}
Corynebacterium glutamicum	ATCC 31833	0.05
"	ATCC 31833/pCPL7	0.72
Corynebacterium herculis	ATCC 13868	0.03
"	ATCC 13868/pCPL7	0.65
Brevibacterium flavum	ATCC 14067	0.04
"	ATCC 14067/pCPL7	0.70
Brevibacterium lactofermentum	ATCC 13869	0.03
"	ATCC 13869/pCPL7	0.64

As can be seen from Table 3. this test showed that the transformants carrying pCPL7 acquired the ability to assimilate lactose.

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(8) Production of glutamic acid from lactose-containing materials

(A) Glutamic acid production test on Corynebacterium glutamicum ATCC 31833, Corynebacterium herculis ATCC I3868, Brevibacterium flavum ATCC I4067 and Brevibacterium lactofermentum ATCC I3869
40 each carrying pCPL7 and prepared in (7) above and their parent strains was carried out in the following manner:
Each strain was inoculated into a seed culture medium (pH 7.2) comprising 40 g/l glucose, 20 g/l polypeptone, I.5 g/l KH₂PO₄, 0.5 g/l K₂HPO₄, 0.5 g/l MgSO4.7H₂O, I0 μg/l biotin and 3 g/l urea, and cultured with shaking at 30°C for 24 hours. For the culture of the transformants. spectinomycin was added
45 to a seed culture medium at a concentration of I00 μg/ml. Then, 4 ml of the seed culture was inoculated into 20 ml of a production medium (pH 6.5) comprising I00 g/l lactose, 2 g/l (NH₄)₂SO₄, I.0 g/l{ KH₂PO₄, 0.5 g/l K₂HPO₄, 0.5 g/l MgSO₄•7H₂O, I mg/l thiamine hydrochloride, 2 mg/l FeSO₄•7H₂O, I0 mg/l MnSO₄•4H₂O, I mg/l CuSO₄•5H₂O, 5 g/l urea and I0 mg/l Phenol Red in a 300 ml-Erlenmeyer flask. Shaking culture was carried out at 30°C for 30 hours, and I ml of I0% urea solution was added I2 hours
50 and 20 hours after the start of culturing to maintain the pH of the culture broth within the range of 6.0 to 8.0. After the culturing was finished. the culture filtrate was subjected to paper chromatography and the amount of L-glutamic acid formed was determined colorimetrically by using the color development with ninhydrin. The results are shown in Table 4.

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Table 4

Strain		L-Glutamic acid (g/ℓ)
Corynebacterium glutamicum	ATCC 31833	0.7
"	ATCC 31833/pCPL7	17.3
Corynebacterium herculis	ATCC 13868	0.5
"	ATCC 13868/pCPL7	11.4
Brevibacterium flavum	ATCC 14067	0.5
"	ATCC 14067/pCPL7	14.6
Brevibacterium lactofermentum	ATCC 13869	0.4
"	ATCC 13869/pCPL7	13.4

(B) Glutamic acid production test on the same strains as used in (A) was carried out in the same manner as in (A), except that whey powder (lactose content: 75%) was used in place of lactose in the production medium in an amount corresponding to l0% lactose, that 5 units/mℓ penicillin G was added when the seed culture was inoculated, and that shaking culture was carried out at 30°C for 72 hours. The amounts of L-glutamic acid determined in the same manner as in (A) are shown in Table 5.

Table 5

Strain		L-Glutamic acid (g/ℓ)
Corynebacterium glutamicum	ATCC 31833	0.6
"	ATCC 31833/pCPL7	15.9
Corynebacterium herculis	ATCC 13868	0.5
"	ATCC 13868/pCPL7	10.2
Brevibacterium flavum	ATCC 14067	0.4
"	ATCC 14067/pCPL7	13.7
Brevibacterium lactofermentum	ATCC 13869	0.4
"	ATCC 13869/pCPL7	12.8

(9) Production of glutamine from lactose-containing material

Corynebacterium glutamicum ATCC l376l was transformed with pCPL7 by the same method as in (7) above, and it was cofirmed that the transformant thus obtained carried pCPL7.

Glutamine production test on the transformant and the parent strain was carried out in the following

manner:

Each strain was inoculated into a seed culture medium (pH 7.2) comprising 50 g/l glucose. 5 g/l (NH₄)₂SO₄. 3 g/l urea, 2 g/l corn steep liquor. 2 g/l meat extract. 0.5 g/l KH₂PO₄. 1.5 g/l K₂HPO₄. 0.5 g/l MgSO₄. 0.02 g/l FeSO₄•7H₂O. 0.02 g/l MnSO₄•7H₂O, 20 µg/l biotin and l mg/l thiamine hydrochloride. and cultured with shaking at 28°C for 24 hours. For the culture of the transformant, spectinomycin was added to a seed culture medium at a concentration of l00 ug/ml. Then, l ml of the seed culture was inoculated into 20 ml of a production medium (pH 6.8) comprising l20 g/l lactose. 40 g/l (NH₄)₂SO₄. 5 g/l urea. 0.5 g/l KH₂PO₄. 0.5 g/l K₂HPO₄. 0.5 g/l MgSO₄•7H₂O. 0.02 g/l FeSO₄•7H₂O. 0.0l g/l MnSO₄•7H₂O. 3.5 ug/l biotin, l mg/l thiamine hydrochloride and 20 g/l CaCO₃ in a 300 ml-Erlenmeyer flask. Shaking culture was carried out at 30°C for 72 hours. After the culturing was finished, the culture filtrate was subjected to paper chromatography and the amount of L-glutamine formed was determined colorimetrically by using the color development with ninhydrin. The results are shown in Table 6.

Table 6

Strain	L-Glutamine (g/l)
Corynebacterium glutamicum ATCC 13761	0.1
" ATCC 13761/pCPL7	3.7

(l0) Production of lysine from lactose-containing materials

(A) Corynebacterium glutamicum RH6 (FERM BP-704) (homoserine-requiring), Brevibacterium flavum ATCC 2l528 (thialysine-resistant) and Brevibacterium lactofermentum ATCC 2l086 (threonine-, isoleucine- and valine-requiring) were each transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformants thus obtained carried pCPL7.

Lysine production test on the transformants and their parent strains was carried out in the following manner:

Each strain was cultured in NB medium at 30°C for l6 hours. For the culture of the transformants, spectinomycin was added to NB medium at a concentration of l00 µg/ml. Then, 0.5 ml of the seed culture was inoculated into 5 ml of a production medium (pH 7.2) comprising l00 g/l lactose, 30 g/l (NH₄)₂SO₄, 0.5 g/l KH₂PO₄, 0.5 g/l K₂HPO₄, l g/l MgSO₄•7H₂O, l0 mg/l FeSO₄•7H₂O, l0 mg/l MnSO₄•7H₂O, l00 µg/l biotin and 30 g/l CaCO₃ in a test tube. Homoserine (l00 µg/ml was further added to the production medium for RH6 strain and its transformant, and l00 µg/ml each of threonine, isoleucine and valine were added to the production medium for ATCC 2l086 strain and its transformant. Shaking culture was carried out at 30°C for 72 hours. After the culturing was finished, the amount of L-lysine formed was colorimetrically determined by the acidic copper ninhydrin method [Chinard, J. Biol. Chem., 199, 9l (1952)]. The results are shown in Table 7.

Table 7

Strain		L-Lysine (g/ℓ)
Corynebacterium glutamicum	RH6 (FERM BP-704)	0.4
"	RH6/pCPL7	7.2
Brevibacterium flavum	ATCC 21528	0.3
"	ATCC 21528/pCPL7	4.8
Brevibacterium lactofermentum	ATCC 21086	0.3
"	ATCC 21086/pCPL7	5.2

(B) Lysine production test on the same strains as used in (A) was carried out in the same manner as in (A), except that whey powder was used in place of lactose in the production medium in an amount corresponding to l00 g/ℓ lactose. The results are shown in Table 8.

Table 8

Strain		L-Lysine (g/ℓ)
Corynebacterium glutamicum	RH6 (FERM BP-704)	0.3
"	RH6/pCPL7	5.5
Brevibacterium flavum	ATCC 21528	0.2
"	ATCC 21528/pCPL7	3.6
Brevibacterium lactofermentum	ATCC 21086	0.3
"	ATCC 21086/pCPL7	3.8

(II) Production of threonine from lactose-containing materials

(A) Corynebacterium glutamicum ATCC 2l660 (methionine-requiring, and resistant to thialysine and α-amino-β-hydroxyvaleric acid) was transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained carried pCPL7.

Threonine production test on the transformant and the parent strain was carried out in the following manner:

Each strain was cultured with shaking in NB medium at 30°C for 24 hours. For the culture of the transformant, spectinomycin was added to NB medium at a concentration of l00 μg/mℓ. Then, 0.5 mℓ of the seed culture was inoculated into 5 mℓ of a production medium (pH 7.2) comprising l00 g/ℓ lactose, 20 g/ℓ $(NH_4)_2SO_4$, 0.5 g/ℓ KH_2PO_4, 0.5 g/ℓ K_2HPO_4, l g/ℓ $MgSO_4•7H_2O$, l0 mg/ℓ $FeSO_4•7H_2O$, l0 mg/ℓ $MnSO_4•7H_2O$, l00 μg/ℓ biotin, 20 g/ℓ $CaCO_3$ and 50 mg/ℓ methionine in a test tube. Shaking culture was carried out at 30°C for 82 hours. After the culturing was finished, the culture filtrate was subjected to paper chromatography and the amount of L-threonine formed was determined colorimetrically by using the color development with ninhydrin. The results are shown in Table 9.

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Table 9

Strain	L-Threonine (g/ℓ)
Corynebacterium glutamicum ATCC 21660	0.1
" ATCC 21660/pCPL7	3.0

(B) Threonine production test on the same strains as used in (A) was carried out in the same manner as in (A), except that whey powder was used in place of lactose in the production medium in an amount corresponding to 100 g/ℓ lactose. The results are shown in Table 10.

Table 10

Strain	L-Threonine (g/ℓ)
Corynebacterium glutamicum ATCC 21660	0.1
" ATCC 21660/pCPL7	2.5

(12) Production of isoleucine from lactose-containing material

Corynebacterium glutamicum N-222 (KY 10589) (FERM P-2843) (lysine-requiring and thiaisoleucine-resistant) was transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained carried pCPL7.

Isoleucine production test on the transformant and the parent strain was carried out in the following manner:

Each strain was cultured with shaking in a seed medium (pH 7.4) comprising 20 g/ℓ glucose, 10 g/ℓ peptone, 10 g/ℓ yeast extract and 2.5 g/ℓ NaCl at 30°C for 24 hours. For the culture of the transformant, spectinomycin was added to a seed medium at a concentration of 100 μg/mℓ. Then, 2 mℓ of the seed culture was inoculated into 20 mℓ of a production medium (pH 7.4) compirisng 100 g/ℓ (as lactose) whey powder, 50 g/ℓ $(NH_4)_2SO_4$, 1 g/ℓ $MgSO_4 \cdot 7H_2O$, 0.5 g/ℓ KH_2PO_4, 0.5 g/ℓ K_2HPO_4, 0.01 g/ℓ $FeSO_4 \cdot 7H_2O$, 0.01 g/ℓ $MnSO_4 \cdot 4H_2O$, 100 μg/ℓ biotin, 100 mg/ℓ lysine hydrochloride and 20 g/ℓ $CaCO_3$ in a 300 mℓ Erlenmeyer flask. Shaking culture was carried out at 30°C for 96 hours. After the culturing was finished, the culture filtrate was subjected to paper chromatography and the amount of L-isoleucine formed was determined colorimetrically by using the color development with ninhydrin. The results are shown in Table 11.

Table 11

Strain	L-Isoleucine (g/ℓ)
<u>Corynebacterium glutamicum</u> N-222 (KY-10589) (FERM P-2843)	0.1
" N-222/pCPL7	·5.1

(l3) Production of valine from lactose-containing material

<u>Brevibacterium lactofermentum</u> KY 10614 (FERM P-2925) (thialysine-resistant) was transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained carried pCPL7.

Valine production test on the transformant and the parent strain was carried out in the following manner:

Each strain was cultured in a seed medium (pH 7.2) comprising 50 g/ℓ glucose, 10 g/ℓ peptone, 10 g/ℓ yeast extract, 2.5 g/ℓ NaCl, 3 g/ℓ urea, 50 μg/ℓ biotin and 5 g/ℓ corn steep liquor at 28°C for 24 hours. For the culture of the transformant, spectinomycin was added to a seed medium at a concentration of 100 μg/mℓ. Then, 2 mℓ of the seed culture was inoculated into 20 mℓ of a production medium (pH 6.8) comprising 100 g/ℓ (as lactose) whey powder, 20 g/ℓ (NH$_4$)$_2$SO$_4$, 0.5 g/ℓ MgSO$_4$•7H$_2$O, 2 g/ℓ KH$_2$PO$_4$, 0.01 g/ℓ FeSO$_4$•7H$_2$O, 0.01 g/ℓ MnSO$_4$•4H$_2$O, 50 μg/ℓ biotin and 30 g/ℓ CaCO$_3$ in a 300 mℓ-Erlenmeyer flask. Shaking culture was carried out at 28°C for 72 hours. After the culturing was finished, the culture filtrate was subjected to paper chromatography and the amount of L-valine formed was determined colorimetrically by using the color development with ninhydrin. The results are shown in Table l2.

Table 12

Strain	L-Valine (g/ℓ)
<u>Brevibacterium lactofermentum</u> KY-10614 (FERM P-2925)	0.2
" KY-10614/pCPL7	7.6

(l4) Production of leucine from lactose-containing material

<u>Brevibacterium lactofermentum</u> ATCC 2l888 (FERM P-l837, thialysine-resistant) was transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained carried pCPL7.

Leucine production test on the transformant and the parent strain was carried out under the same conditions as in the valine production test described in (l3) above. The results are shown in Table l3.

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Table 13

Strain	L-Leucine (g/ℓ)
Brevibacterium lactofermentum ATCC 21888	0.1
" ATCC 21888/pCPL7	4.9

(I5) Production of tryptophan from lactose-containing materials

(A) Corynebacterium glutamicum K-55 (FERM BP-864) (phenylalanine-requiring, tyrosine-requiring, and resistant to 5-methyltryptophan, tryptophan hydroxamate, 6-fluorotryptophan, 4-methyltryptophan, p-fluorophenylalanine, p-aminophenylalamine, tyrosine hydroxamate and phenylalanine hydroxamate) was transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained carried pCPL7.

Tryptophan production test on the transformant and the parent strain was carried out in the following manner:

Each strain was cultured with shaking in NB medium at 30°C for 16 hours. For the culture of the transformant, spectinomycin was added to NB medium at a concentration of 100 ug/mℓ. Then, 0.5 mℓ of the seed culture was inoculated into 5 mℓ of a production medium (pH 7.2) comprising 100 g/ℓ lactose, 20 g/ℓ - $(NH_4)_2SO_4$, 0.5 g/ℓ KH_2PO_4, 0.5 g/ℓ K_2HPO_4, 0.25 g/ℓ $MgSO_4•7H_2O$, 2.5 g/ℓ NZ-amine and 20 g/ℓ $CaCO_3$ in a test tube. Shaking culture was carried out at 30°C for 84 hours. After the culturing was finished, the culture filtrate was subjected to paper chromatography and the amount of L-tryptophan formed was determined colorimetrically by using the color development with ninhydrin. The results are shown in Table I4.

Table 14

Strain	L-Tryptophan (g/ℓ)
Corynebacterium glutamicum K-55 (FERM BP-864)	0.2
" K-55/pCPL7	2.8

(B) Tryptophan production test on the same strains as used in (A) was carried out in the same manner as in (A), except that whey powder was used in place of lactose in the production medium in an amount corresponding to 100 g/ℓ lactose. The results are shown in Table I5.

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Table 15

Strain	L-Tryptophan (g/ℓ)
Corynebacterium glutamicum K-55 (FERM BP-864)	0.1
" K-55/pCPL7	2.4

(16) Production of phenylalanine from lactose-containing material

Corynebacterium glutamicum K38 (FERM BP-454) (resistant to p-fluorophenylalanine and p-aminophenylalanine) was transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained carried pCPL7.

Phenylalanine production test on the transformant and the parent strain was carried out under the same conditions as in the tryptophan production test described in (I5)-(B) above. The results are shown in Table I6.

Table 16

Strain	L-Phenylalanine (g/ℓ)
Corynebacterium glutamicum K38 (FERM BP-454)	0.1
" K38/pCPL7	2.8

(I7) Production of tyrosine from lactose-containing material

Corynebacterium glutamicum K43 (FERM BP-457) (resistant to 3-aminotyrosine, p-aminophenylalanine, p-fluorophenylalanine and tyrosine hydroxamate) was transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained carried pCPL7.

Tyrosine production test on the transformant and the parent strain was carried out under the same conditions as in the tryptophan production test described in (I5)-(B) above. The results are shown in Table I7.

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Table 17

Strain	L-Tyrosine (g/ℓ)
Corynebacterium glutamicum K43 (FERM BP-457)	0.1
" K43/pCPL7	2.0

(18) Production of histidine from lactose-containing material

Corynebacterium glutamicum H-d (FERM P-8185) (resistant to 2-thiazolalanine, 6-mercaptoguanine, 8-azaguanine, 6-azauracil, 6-methylpurine, 5-methyltryptophan, streptomycin, gramicidin and p-coumaric acid) was transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained carried pCPL7.

Histidine production test on the transformant and the parent strain was carried out in the following manner:

Each strain was cultured with shaking in a seed medium (pH 7.2) comprising 40 g/ℓ glucose, 20 g/ℓ polypeptone, 1.5 g/ℓ KH$_2$PO$_4$, 0.5 g/ℓ K$_2$HPO$_4$, 0.5 g/ℓ MgSO$_4$•7H$_2$O, 50 μg/ℓ biotin, 5 g/ℓ yeast extract, 3 g/ℓ urea and 10 mg/ℓ thiamine hydrochloride at 30°C for 24 hours. For the culture of the transformant, spectinomycin was added to a seed medium at a concentration of 100 μg/mℓ. Then, 1 mℓ of the seed culture was inoculated into 20 mℓ of a production medium (pH 7.4) comprising 70 g/ℓ (as lactose) whey powder, 5 g/ℓ meat extract, 40 g/ℓ (NH$_4$)$_2$SO$_4$, 0.5 g/ℓ MgSO$_4$•7H$_2$O, 1.5 g/ℓ KH$_2$PO$_4$, 0.5 g/ℓ K$_2$HPO$_4$, 3 g/ℓ urea, 2.5 g/ℓ NaCl, 10 mg/ℓ FeSO$_4$•7H$_2$O, 10 mg/ℓ MnSO$_4$•4H$_2$O, 2 mg/ℓ CuSO$_4$, 1 mg/ℓ ZnSO$_4$, 100 μg/ℓ biotin, 10 mg/ℓ thiamine hydrochloride, 10 mg/ℓ β-alanine, 10 mg/ℓ nicotinic acid, 10 mg/ℓ calcium pantothenate and 30 g/ℓ CaCO$_3$ in a 300 mℓ-Erlenmeyer flask. Shaking culture was carried out at 30°C for 84 hours. After the culturing was finished, the culture filtrate was subjected to paper chromatography and the amount of L-histidine formed was determined colorimetrically with Pauly's reagent by using the color development with ninhydrin [Agric. Biol. Chem., 38, 189 (1974)]. The results are shown in Table 18.

Table 18

Strain	L-Histidine (g/ℓ)
Corynebacterium glutamicum H-d (FERM P-8185)	0.4
" H-d/pCPL7	6.7

(19) Production of arginine from lactose-containing material

Arginine production test from a lactose-containing material was carried out by using Corynebacterium glutamicum ATCC 31833 carrying pCPL7 and plasmid pEarg5 containing the genes responsible for arginine-synthesis.

The plasmid pEarg5 was prepared from plasmid pEargI (Japanese published Unexamined Patent Application No. 66989/85) containing genes responsible for arginine-synthesis. argECBH genes derived from Escherichia coli according to the procedure described below.

Plasmid pEargI DNA was isolated from Corynebacterium glutamicum K46 (FERM BP-356) in the following manner. That is, the strain was cultured with shaking in SSM medium at 30°C. When OD reached 0.2. penicillin G was added to the culture medium to a concentration of 0.5 unit·mℓ, and the culturing was continued until OD reached 0.6. From the cultured cells thus obtained. pEargI plasmid DNA was isolated in the same manner as in (2) above for the isolation of pCGII.

Then, 2 µg of the plasmid DNA thus isolated was dissolved in 20 µℓ of reaction buffer solution for restriction enzyme SalI (10mM Tris, 10mM MgCl₂, 150mM NaCl and 1mM dithiothreitol, pH 7.5), and 6 units each of Pst I and SalI were added thereto. The mixture was subjected to reaction at 37°C for 60 minutes. The reaction mixture was subjected to agarose gel electrophoresis, and about 0.3 µg of a SalI-PstI-cleaved DNA fragment of 4.0 kb containing argBCH genes was obtained by the method of Girvitz, et al.

Separately. plasmid pCE54 (Japanese Published Unexamined Patent Application No. 105999/83) autonomously replicable in both Escherichia coli and strains of the genus Corynebacterium was isolated from Escherichia coli KI2 strain carrying pCE54 by the method of Ann, et al. To 20 µℓ of reaction buffer solution for SalI containing 5 µg of pCE54 plasmid DNA, was added 6 units of SalI, and the mixture was subjected to reaction at 37°C for 60 minutes. Further, 2 units of PstI was added thereto, and the mixture was subjected to reaction at 37°C for 30 minutes. About 1 µg of a SalI-PstI-cleaved DNA fragment of 10.6 kb was isolated from the reaction mixture by the method fo Girvitz, et al.

Both DNA fragments obtained above were dissolved in 180 µℓ of TES buffer solution, and 20 µℓ of buffer solution for T4 ligase at a 10-fold concentration, 2 µℓ of 100mM ATP and 350 units of T4 ligase were added thereto. The mixture was subjected to ligase reaction at 12°C for 16 hours. The ligase reaction mixture thus obtained was used for the transformation of Escherichia coli K294 by the method of Dagert, et al., using L-agar medium containing 20 µg/mℓ kanamycin as a selection medium. The colonies grown on the medium were selected. The transformants thus obtained were spread on L-agar medium containing 25 µg/mℓ chloramphenicol, and the colonies which did not grow thereon were selected. A plasmid was isolated from the transformant thus obtained by the alkali method, and after digestion with restriction enzymes, the plasmid was analyzed by agarose gel electrophoresis. It was found that the plasmid had a structure wherein the DNA fragment of 4.0 kb derived from pEargI and the DNA fragment of 10.6 kb derived from pCE54 and containing the kanamycin-resistance gene were joined at their PstI-and SalI-cohesive ends. This plasmid was named pEarg5.

Plasmid pEarg5 DNA thus prepared was used to transform Corynebacterium glutamicum ATCC 31833, and arginine production test on the transformant was carried out in the following manner:

The transformation was carried out by the protoplast method described in (2) using RCGP agar medium containing 200 µg/mℓ kanamycin as a selection medium. The transformant thus obtained was cultured with shaking in NB medium containing 20 ug/mℓ kanamycin at 30°C for 16 hours. Then, 0.5 mℓ of the seed culture was inoculated into 5 mℓ of a production medium (H 7.0) comprising 80 g/ℓ (as glucose) blackstrap molasses. 40 g/ℓ (NH₄)₂SO₄, 0.5 g/ℓ KH₂PO₄, 0.5 g/ℓ K₂HPO₄ and 20 g/ℓ CaCO₃ in a test tube. Shaking culture was carried out at 30°C for 72 hours. After the culturing was finished, the culture filtrate was subjected to paper chromatography and the amount of L-arginine formed was determined colorimetrically by using the color development with ninhydrin. The amount of L-arginine produced was 1.5 g/ℓ, and thus pEarg5 was confirmed to be capable of conferring the ability to produce arginine on glutamic acid-producing coryneform bacteria.

Corynebacterium glutamicum ATCC 31833 carrying pEarg5 was further transformed with pCPL7 by the same method as in (7) above. That is, the transformation was carried out by the protoplast method described in (2) using RCGP agar medium containing 200 µg/mℓ kanamycin and 400 µg/mℓ spectinomycin as a selection medium. It was confirmed that the transformant obtained carried both pEarg5 and pCPL7.

Arginine production test on the strain carrying pEarg5 and the strain carrying both pCPL7 and pEarg5 was carried out in the following manner:

Each strain was cultured with shaking in NB medium containing 20 µg/mℓ kanamycin for the strain carrying pEarg5. and 20 µg/mℓ kanamycin and 100 µg/mℓ spectinomycin for the strain carrying both pCPL7 and pEarg5 at 30°C for 16 hours.

Then, 0.5 mℓ of the seed culture was inoculated into 5 mℓ of a production medium (pH 7.0) comprising 80 g/ℓ (as lactose) whey powder, 40 g/ℓ (NH₄)₂SO₄, 0.5 g/ℓ KH₂PO₄, 0.5 g/ℓ K₂HPO₄ and 20 g/ℓ CaCO₃ in a test tube. Shaking culture was carried out and the amount of L-arginine formed was determined in the same manner as above. The results are shown in Table 19.

Table 19

Strain	L-Arginine (g/ℓ)
Corynebacterium glutamicum ATCC 31833/pEarg5	0.01
" ATCC 31833/pEarg5, pCPL7	0.6

(20) Production of ornithine from lactose-containing material

Corynebacterium glutamicum ATCC 13232 (arginine-requiring) was transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained carried pCPL7.

Ornithine production test on the transformant and the parent strain was carried out in the following manner:

Each strain was cultured with shaking in NB medium at 30°C for 16 hours. For the culture of the transformant, spectinomycin was added to NB medium at a concentration of 100 μg/mℓ. Then, 0.5 mℓ of the seed culture was inoculated into 5 mℓ of a production medium (pH 7.0) comprising 80 g/ℓ lactose, 40 g/ℓ $(NH_4)_2SO_4$, 0.5 g/ℓ KH_2PO_4, 0.5 g/ℓ K_2HPO_4, 100 mg/ℓ L-arginine and 20 g/ℓ $CaCO_3$ in a test tube, and shaking culture was carried out at 30°C for 72 hours. After the culturing was finished, the culture filtrate was subjected to paper chromatography and the amount of L-ornithine formed was determined colorimetrically by using the color development with ninhydrin. The results are shown in Table 20.

Table 20

Strain	L-Ornithine (g/ℓ)
Corynebacterium glutamicum ATCC 13232	0.05
" ATCC 13232/pCPL7	9.2

(21) Production of citrulline from lactose-containing material

Corynebacterium glutamicum K61 (FERM BP-IIII) (arginine-requiring) was transformed with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained carried pCPL7.

Citrulline production test on the transformant and the parent strain was carried out under the same conditions as in the ornithine production test described in (20) above. The results are shown in Table 21.

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Table 21

Strain	L-Citrulline (g/ℓ)
Corynebacterium glutamicum K61 (FERM BP-1111)	0.02
" K61/pCPL7	0.5

(22) Production of proline from lactose-containing material

    Corynebacterium glutamicum H-3334 (FERM P-6823) (6-mercaptoguanosine-resistant) was transformed
with pCPL7 by the same method as in (7) above, and it was confirmed that the transformant thus obtained
carried pCPL7.
    Proline production test on the transformant and the parent strain was carried out in the following
manner:
    Each strain was cultured with shaking in a seed medium (pH 7.2) comprising 10 g/ℓ glucose, 5 g/ℓ meat
extract, 10 g/ℓ peptone, 3 g/ℓ yeast extract and 3 g/ℓ NaCl at 30°C for 24 hours. For the culture of the
transformant, spectinomycin was added to a seed medium at a concentration of 100 μg/mℓ. Then, 2 mℓ of
the seed culture was inoculated into 20 mℓ of a production medium (pH 7.4) comprising 100 g/ℓ (as
lactose) whey powder, 10 g/ℓ (NH₄)₂SO₄, 3 g/ℓ KH₂PO₄, 0.5 g/ℓ MgSO₄•7H₂O, 10 mg/ℓ FeSO₄•7H₂O, 10 mg/ℓ
nicotinic acid, 100 μg/ℓ thiamine hydrochloride, 100 μg/ℓ biotin, 20 g/ℓ corn steep liquor, 20 g/ℓ sodium L-
glutamate and 30 g/ℓ CaCO₃ in a 300 mℓ-Erlenmeyer flask, and shaking culture was carried out at 32°C for
96 hours. After the culturing was finished, the amount of L-proline formed in the culture filtrate was
determined by the method of Chinard [J. Biol. Chem., 199, 91 (1952)]. The results are shown in Table 22.

Table 22

Strain	L-Proline (g/ℓ)
Corynebacterium glutamicum H-3334 (FERM P-6823)	0.2
" H-3334/pCPL7	5.1

Claims

    1. A microorganism belonging to the genus Corynebacterium or Brevibacterium and capable of
assimilating lactose.
    2. A microorganism according to claim 1, wherein the microorganism belongs to the species Corynebac-
terium glutamicum, Corynebacterium herculis, Brevibacterium flavum or Brevibacterium lactofermentum.
    3. A microorganism according to claim 1, wherein the microorganism carries a recombinant DNA which
confers the ability to assimilate lactose on microorganisms belonging to the genus Corynebacterium or
Brevibacterium, said recombinant DNA comprising (a) a part or all of the coding region of the structural

genes for β-D-galactosidase and β-galactoside permease, which is derived from Escherichia coli lactose operon, and (b) a DNA fragment derived from a microorganism belonging to the genus Corynebacterium or Brevibacterium and enabling expression of foreign genes in strains belonging to the genus Corynebacterium or Brevibacterium , which is positioned upstream of the coding region of said structural genes.

4. A process for producing amino acids which comprises culturing a microorganism belonging to the genus Corynebacterium or Brevibacterium and capable of assimilating lactose in a culture medium containing lactose to form an amino acid, and recovering said amino acid accumulated in the culture broth.

5. A process for producing amino acids according to claim 4, wherein said microorganism carries a recombinant DNA which confers the ability to assimilate lactose on microorganisms belonging to the genus Corynebacterium or Brevibacterium, said recombinant DNA comprising (a) a part or all of the structural genes coding for β-D-galactosidase and β-galactoside permease, which is derived from Escherichia coli lactose operon, and (b) a DNA fragment derived from a microorganism belonging to the genus Corynebacterium or Brevibacterium and enabling genes to be expressed in strains belonging to the genus Corynebacterium or Brevibacterium, which is positioned upstream of the coding region of said structural genes.

6. A process for producing amino acids according to claim 4, wherein said amino acid is glutamic acid, glutamine, lysine, threonine, isoleucine, valine, leucine, tryptophan, phenylalanine, tyrosine, histidine, arginine, ornithine, citrulline or proline.

7. A recombinant DNA which confers the ability to assimilate lactose on microorganisms belonging to the genus Corynebacterium or Brevibacterium, said recombinant DNA comprising (a) a part or all of the region coding for the structural genes for β-D-galactosidase and β-galactoside permease, which is derived from Escherichia coli lactose operon, and (b) a DNA fragment derived from a microorganism belonging to the genus Corynebacterium or Brevibacterium and enabling genes to be expressed in strains belonging to the genus Corynebacterium or Brevibacterium, which is positioned upstream of the coding region of said structural genes.

8. Plasmid pCPL7 (FERM BP-II79).

9. A plasmid which has autonomous replicability in a microorganism belonging to the genus Corynebacterium or Brevibacterium and contains selectable genetic markers, a DNA fragment derived from Escherichia coli lactose operon and carrying the coding region of a part or all of structural genes necessary for the expression of β-D-galactosidase activity, and restriction enzyme cleavage sites positioned just before said coding region.

10. Plasmid pE'lacI (FERM BP II78).

II. A method for preparing a recombinant plasmid containing a DNA fragment responsible for the initiation of transcription and translation necessary for the expression of a gene in a microorganism belonging to the genus Corynebacterium or Brevibacterium, which comprises (a) inserting a DNA fragment derived from a microorganism belonging to the genus Corynebacterium or Brevibacterium into a plasmid at a restriction enzyme cleavage site, said plasmid having autonomous replicability in a microorganism of the genus Corynebacterium or Brevibacterium, and containing selectable genetic markers, a DNA fragment derived from Escherichia coli lactose operon and carrying the coding region of structural genes necessary for the expression of β-D-galactosidase activity, and restriction enzyme cleavage sites positioned just before said coding region; (b) transforming a strain of the genus Corynebacterium or Brevibacterium by using the recombinant plasmid thus prepared to obtain a transformant having the ability to assimilate lactose; and (c) isolating the recombinant plasmid from said transformant.
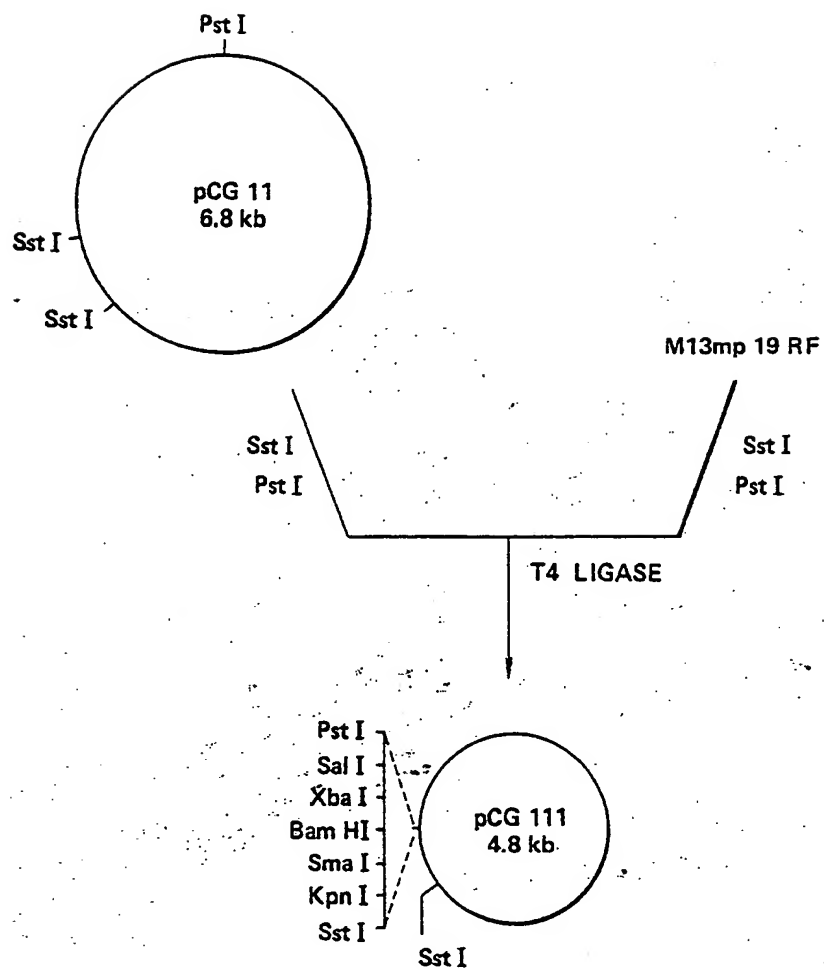
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FIG. 1

FIG.2